AD_____

Award Number: W81XWH-12-1-0565

TITLE: Intravesical NGF Antisense Therapy Using Lipid Nanoparticle For Interstitial Cystitis

PRINCIPAL INVESTIGATOR: Michael Chancellor

CONTRACTING ORGANIZATION: Lipella Pharmaceuticals, Inc.
Pittsburgh, PA 15208

REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	30September2012-29September2013

4. TITLE AND SUBTITLE
Intravesical NGF Antisense Therapy Using Lipid Nanoparticle For Interstitial Cystitis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0565

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Michael Chancellor

chancellormb@gmail.com

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lipella Pharmaceuticals, Inc.
Pittsburgh, PA 15208

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The Technology Therapeutic Development Award titled "Intravesical NGF antisense therapy using lipid nano-particle for interstitial cystitis" evaluates the feasibility of an anti-nerve growth factor (NGF) bladder drug delivery system targeting Interstitial Cystitis/Painful Bladder Syndrome (IC/PBS), IC/PBS is a chronic, severely debilitating disease of the urinary bladder. The goal of this project is to advance key preclinical experiments towards the development of a new drug. Specific aims include experiments, manufacture, and animal testing of experimentsal formulations. During the first year of the project, progress has been made according to the statement of work. Advancements in the development of product formulation and completion of initial manufacturing runs were accomplished. Progress on the timeline towards study drug investigation in advanced diseased animal models is on schedule.

15. SUBJECT TERMS- interstitial cystitis, painful bladder syndrome, liposome, nerve growth factor, afferent hyper-excitability, antisense

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS:

I. Introduction

Interstitial cystitis/painful bladder syndrome (IC/PBS) is a poorly understood condition characterized by chronic inflammation of the bladder wall. IC/PBS is associated with bladder symptoms of urinary frequency and urgency, and is pain. IC/PBS has a prevalence range between 700,000 and 1 million Americans. This project will investigate the feasibility of a new local-treatment of liposome mediated delivery of anti-nerve growth factor (NGF) to address IC/PBS. Aims of the project include manufacture and optimization of the new product, leading to preclinical studies in diseased animal models. Neither the proprietary name nor the unique prefix for the non-proprietary name has been assigned to this investigative product, at this time we refer to formulations as LP-11.

II. Body

	Year 1			Year 2			Year 3		
	0-4mo	5-8mo	9-12mo	13-16mo	17-20mo	21-24mo	25-28mo	29-32mo	33-36mo
AIM 1	□ Regulatory approval for animal research □ Obtain written approval from both IACUC and ACURO	□ Order animals/ □ Prepare protocols/ documentation □ Initiate animal study and confirmation of CYP and TNBS models		□ CYP, TNBS treatment □ LP-NGF antisense treatment Pain behavior/ bladder over-activity assessment □ Tissue analysis o PCR/ ELISA o Patch Clamp			Complete animal studies		Animal study data analysis/ reporting
AIM 2	• Optimize formulation • Manufacture LP • Analytical method development			• Develop GMP LP-NGF antisense manufacture process • cGMP documentation/reporting • Manufacture GMP LP • Product specifications /batch release testing • Biodistribution assessment			• Stability Testing • Ex-Vivo Stress Testing		

AIM 1(Year 1) *Regulatory approval for animal research; Obtain written approval from both IACUC and ACURO*

[Accomplishment]

IACUC approval was earned for protocol number: 1201267, titiled "Intravesical NGF Antisense Therapy Using Lipid Nanoparticle for Intersitital Cystitis" with assurance number A3187-01 in January 2012 and renewed in January, 2013 by the University of Pittsburgh Institutional Animal Care and Use Committee. Furthermore, ACURO renewal for approval of the same protocol was confirmed on Thursday, January 10, 2013

AIM 1 (Year 1) *Order animals/ Prepare protocols/ documentation Initiate animal study and confirmation of CYP and TNBS models*

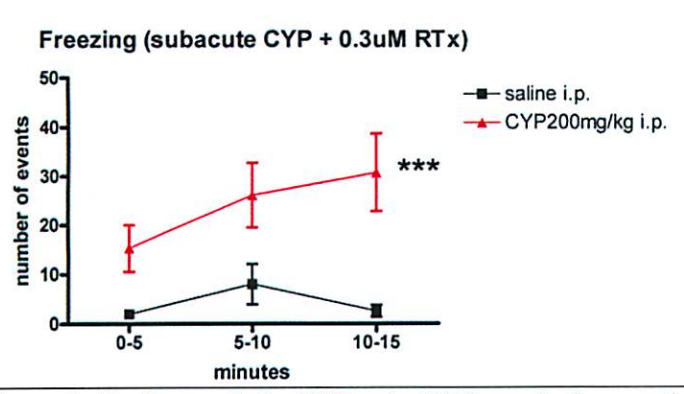
[Accomplishment]

1. Confirmation of CYP cystitis model

During the time of this grant, we examined whether chemical cystitis induced by cyclophosphamide (CYP) enhance nociceptive responses to resiniferatoxin (RTx) stimulation to confirm that the CYP model is suitable for bladder pain research. Saline or CYP200mg/kg was injected intraperitoneally to female SD rats. Two days later, in an awake condition, 0.3μM RTx (0.3ml, 1 min) was injected into the bladder through a urethral catheter to evaluate nociceptive behaviors such as freezing (motionless head-turning) that were counted and recorded every 5 seconds for 15 minutes. Urine volume and frequency were recorded simultaneously in metabolic cages (n=5 each).

We have found that: (1) freezing behavior, which reflects bladder pain, induced by RTx was significantly increased in CYP cystitis vs. control rats and (2) bladder volume (urine volume/ micturition) was significantly

decreased in CYP cystitis vs. control rats (Fig. 1). These results indicated that CYP rats have bladder pain associated with frequent voiding, which are the main symptoms of IC/BPS.



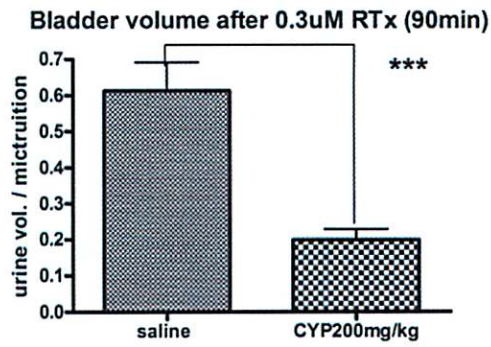


Figure 1. [Left panel] In CYP rats (48 hours), the number of freezing behavior observed after intravesical application of resiniferatoxin (RTx) was significantly increased, especially during 10-15 min after RTx instillation. [Right panel] In CYP rats (48 hours), bladder volume (urine volume/ micturition) after intravesical RTx application was significantly smaller compared to saline-treated rats without cystitis, ***p<0.001

2. Confirmation of TNBS colitis model

[Accomplishment]

During the time of this grant, we also examined whether pain behavior in response to bladder irritation is enhanced in rats with experimental chronic colitis to confirm that the model is suitable for bladder pain research. Experimental colitis was induced by intracolonic injection of trinitrobenzene sulfonic acid (TNBS 50 mg/mL in 50% ethanol, 0.4mL) through a transanally placed. Ten days later, in an awake condition, 0.3μM RTx (0.3ml, 1 min) was injected into the bladder through a urethral catheter to evaluate nociceptive behaviors such as freezing that were counted and recorded every 5 seconds for 20 minutes (n=5 each).

We have found that; (1) freezing behavior, which reflects bladder pain, was significantly increased in TNBS colitis vs. control rats, (2) increased freezing behavior in colitis rats was suppressed when C-fiber afferent pathways were desensitized by capsaicin pretreatment (Fig. 2) and (3) the NGF protein level in the mucosa containing urothelial cells was significantly ($p<0.05$) increased in colitis rats (261.8 pg/mg total protein, n=4) compared to sham rats (136.8 pg/mg, n=5). These results indicated that TNBS colitis rats have bladder pain due to activation of C-fiber afferent pathways, which is associated with NGF upregulation in the mucosa.

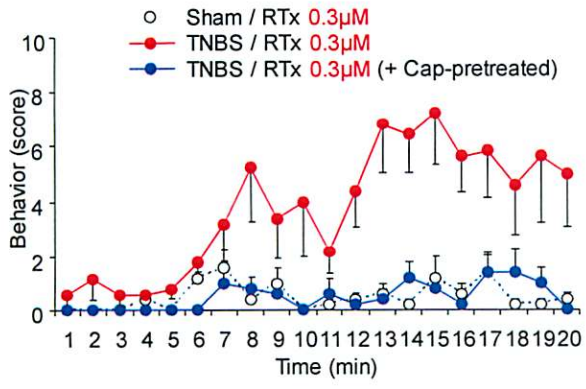


Figure 2. In TNBS-induced colitis rats (TNBS/RTx 0.3μM), the number of freezing behavior observed after intravesical application of resiniferatoxin (RTx) was significantly increased, especially during 10-20 min after RTx instillation, compare to sham rats (Sham/RTx 0.3μM). This enhanced freezing behavior induced by RTx was almost totally abolished in TNBS colitis rats with capsaicin pretreatment (125mg/kg, s.c., 4 days earlier).

AIM 2(Year 1) *Optimize formulation*

[Accomplishment]

Optimization of intravesical liposome-NGF antisense treatment

During the time of this grant, we optimized and manufactured liposomes (LPs) conjugated with NGF antisense as follows. The 18mer phosphorothioate oligodeoxynucleotide (ODN) with the sequence 5'-GCCCGAGACGCCTCCCGA-3' for the experiments were made, and cationic liposomes composed of DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N trimethylammonium methylsulfate) were made by thin film hydration method and hydrated with nuclease free water with the final lipid concentration of 7mM. The ODN were dissolved in nuclease free water at the concentration of 2mM and were complexed with liposomes in the proportion of 6μl ODN solution to 1 ml liposome lipid by incubation at room temperature for 30min.

Then, we perform in-vivo experiments to test the efficacy of LPs conjugated with NGF antisense. Under isoflurane anesthesia, rats were catheterized by a 24-gauge angiocatheter through the urethra into the bladder. After urine was drained from the bladder, 12μM of NGF antisense or scramble ODN complexed with liposome or saline in a volume of 0.5ml was infused for 30 minutes. The efficacy of LP-antisense treatments was assessed 24h after infusion by saline and subsequent acetic acid (AA) cystometry under urethane anesthesia. A control cystometrogram (CMG) was performed during filling the bladder with saline to elicit repetitive voiding more than for 1 hour followed by 0.25% acetic acid (AA) infusion to induce bladder irritation for more than 3 hours. The intercontractile interval (ICI) of the reflex bladder contractions during saline and AA was measured and compared.

We have found that; (1) LPs complexed with NGF antisense were retained in the urothelium after intravesical application as evidenced by histological identification of LP antisense tagged with a fluorescent dye (Kashyap et al, 2013), (2) LPs-NGF antisense treatment suppressed AA-induced bladder oveactivity as evidenced by the reduction in the ICI decrease after intravesical AA application in the LPs-NGF-treated groups vs. control groups (saline or LPs-scramble oligo treatment) (Fig. 3), and (3) LPs-NGF antisense treatment reduced the NGF expression in the bladder mucosal layer (Kashyap et al, 2013). These results indicate that the LPs-NGF antisense complex is effective to suppress the urothelial NGF expression and inhibit bladder overactivity induced by bladder afferent sensitization. We will use this technique to investigate whether intravesical LP-NGF antisense treatment can suppress bladder pain in both CYP cystitis and TNBS colitis models.

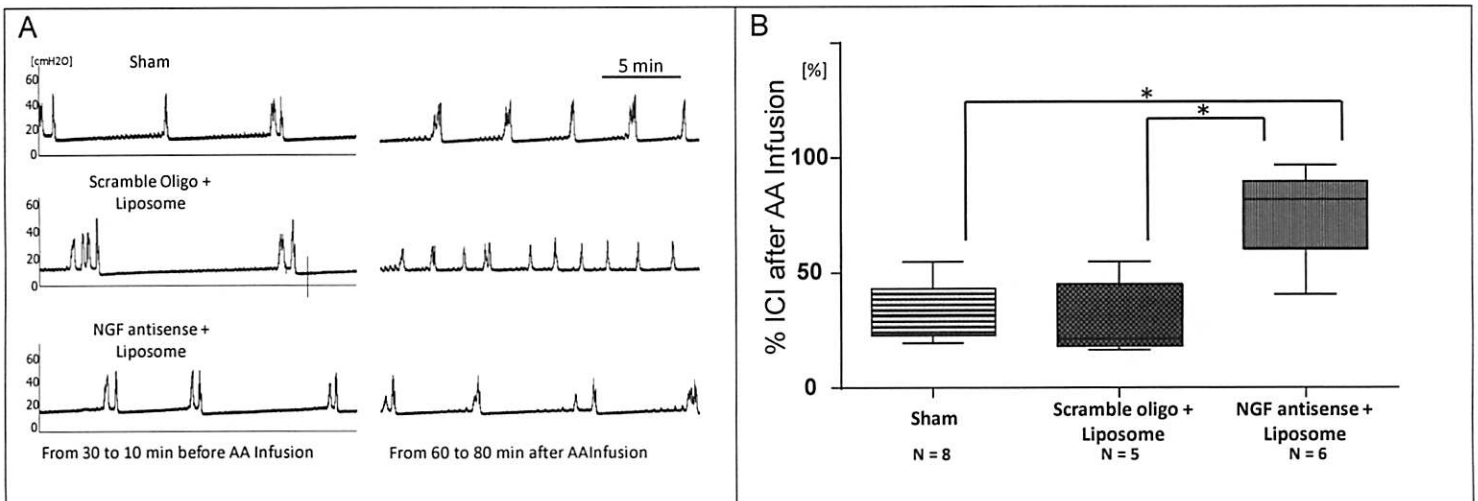


Figure 3. Cystometric analysis of the effects of liposome (LP)-NGF antisense treatment on bladder overactivity induced by intravesical application of acetic acid (AA; 0.25%) in rats. A: Representative cystometrograms showing the effects of intravesical application of AA in sham (saline treatment) (upper traces), LP-scramble oligo-treated (middle traces) and LP-NGF antisense treated rats (lower traces). The cystometrograms before and after are shown in left and right traces, respectively. The AA-induced reduction in intercontraction intervals (ICI) was seen in sham and scramble oligo-treated rats, but not in the rat treated with LP-NGF antisense conjugates. B: Changes in ICIs after intravesical AA application expressed as the percent ratio of ICI after AA application against the ICI values prior to AA application. The AA-induced reduction of ICI was significantly smaller in the LP-NGF antisense treated group (n=6) compared to sham (saline-treated) (n=8) and LP-scramble oligo-treated groups (n=5). *$p<0.05$ compared to sham or scramble oligo groups

AIM 2(Year 1) *Manufacture LP*

[Accomplishment]

During the time of this grant, Lipella has developed a scalable manufacturing method for LP-11. Over the first project period specific procedures for handling, processing, environmental control, identification, packaging, testing, storage, and release of the company's product were created. Standard operating procedures (SOPs) have been put in place for the full manufacturing method. At this time, liposomal processing at Lipella includes aseptic handling under procedurally controlled conditions to prevent contamination and to preserve function and integrity of an LP-11 product.

Over the year, Lipella has worked to secure LP-11 material sourcing with validated material companies for production and testing. Lipella purchases oligonucleotides from Integrated DNA Technologies (IDT). IDT's manufacturing headquarters achieved ISO 9001:2008: Quality Management Systems certification in 2005 and ISO 13485:2003: Medical Device Quality Mangement Systems certification in 2008. Mass spectrometry is used for Quality Control verification of each oligonucleotide synthesized. Lipella purchases the lipids from Avanti Polar Lipids. The sterile water for injection USP is similarly purchased from Baxter Healthcare Corporation. These incoming material requirements assist in controlling the consistency.

To verify a sterile production process Lipella evaluated LP-11 aseptic processing during fill finish and sealing and also conducted production rules verifying sterility of manufacturing environment as well as final product by sterility and endotoxin testing. Evaluation of aseptic manufacturing involves the development of methods to verify a sterile production process of test articles. SOPs to identify and control sources of bio-burden were created, and s SOP for all filters used during the manufacturing process is now reserved for bio-burden testing. Final product is tested in accordance with Lipella SOPs for compliance with final product release specifications. To enhance aseptic capabilities, the Lipella manufacturing suite and adjacent gowning room were upgraded and cleaned with 6% Sterile Hydrogen Peroxide (6% Steri Perox, supplied by Veltek Associates Inc.) with a minimum contact time of 1 hour. All surfaces of cleanroom including walls, floor, furniture and equipment (table, stools), and materials are sprayed except those for which spraying is unsuitable. The inside of the lyophilizer as well as the ceiling containing hepa filters are wiped clean with wipes saturated with 6% Hydrogen Peroxide.

During the first period of this grant, three environmental monitoring and cleaning evaluations were performed using Contact Sterile TSA Plates for surface viable counts; a Met One Particle Counter model no. 804 for non-viable air sampling counts; and Sterile TSA settling plates for viable air counts. Non-viable air counts were measured for 60 seconds and settle plates were exposed for a minimum of 4 hours. Contact and settling plates were incubated at 35°C for 7 days. Selection of environmental monitoring locations was based on a risk assessment. Viable air sampling was performed in the areas immediately adjacent to critical zones where work is being done. Dimensions of the manufacturing room are 5x7x7' with an identical size gowning room adjacent and separated by soft-wall curtain. Due to the small size of the manufacturing and gowning areas, a grid profiling approach to contact plate sampling location and sample number is not appropriate. One contact plate was used for each of the walls, ceiling, floor, furniture, two used on table, and three on product processing equipment (one inside, top and front). Similarly, one contact plate was used for each of the walls, ceiling, floor and table in the gowning room. Contact plates were used to evaluate the sterility of operators' gloved fingertips following production. These assessments were reflected in evaluations results from the studies. No growth was observed on using plates during the three evaluation runs. Additionally, non-viable air sampling returned counts within limits for the classification of the room.

These steps help ensure an acceptable environment for manufacturing LP-11. Furthermore, a class II SterilGAR hood was acquired with the goal to support future manufacturing of LP-11. We anticipate this addition will provide production scale up through anticipated early stage clinical trials of LP-11's development. Ongoing incorporation of equipment during the second project period will improve Lipella's production of LP-11, with intent to be available for use in time for proposed diseased animal model experiments. Finally, Lipella

completed an initial full pilot scale production run following grant developed SOPs for LP-11, successful completing a batch of 8 vials including material for release testing. The work accomplished in budget period 1 will help to make future runs able to support the demand required for production scale up.

AIM 2(Year 1) *Analytical method development*
[Accomplishment]
During the time of this grant, Lipella evaluated shelf life of LP-11 components using laser light scattering to determine particle size distribution of liposomes following freezer storage at -20°C as compared to the particle size distribution post-manufacture. Results from the particle size analysis noted that the test article can remain within release specifications following storage at -20°C. Physical stability of product was also assessed by noting lack of aggregation/precipitation following reconstitution. Additional evaluation of LP-11 shelf life testing with a sample stored at 40°C with relative humidity of 50-75% is ongoing.

Stress stability of liposomes was analyzed under two conditions: reconstitution with sterile water at room temperature and physiological stress conditions (reconstituted liposomes diluted in synthetic urine at 37°C). Separate batches were analyzed using microscopy, at room temperature for four hours to evaluate stability of reconstituted liposomes for clinical use and reconstituted liposomes in urine at 37°C for one hour to evaluate stress stability conditions of the urinary bladder. Microscopy video was taken for two separate batches of liposomes each in water at room temperature (4 hours) and in urine at 37°C (1 hour). Screenshots were taken at the beginning and end of each video and liposomes in both viewing windows were qualitatively compared with regard to size. In general, no observable changes in liposome size were recorded, indicating that the product relatively stable both in room temperature water and 37°C urine throughout the period in which they were tested. Additional stress stability of LP-11 is ongoing.

III. Key research accomplishments

- Preparation and approval of animal protocols/documentation
- Preparation and confirmation of in vivo animal models
- Formulation bladder administration of liposomes-NGF antisense
- Material controls
- Aseptic manufacturing evaluation
- Pilot production of LP11
- Initiation stability and shelf life testing

IV. Reportable outcomes

Publications:
Refereed article:

1. Kashyap M, Kawamorita N, Tyagi V, Sugino Y, Chancellor M, Yoshimura N, Tyagi P.,(2013) Down - Regulation of Nerve Growth Factor Expression in the Bladder by Antisense Oligonucleotides as New Treatment for Overactive Bladder. Journal of Urology, 190: 757-764

Published abstracts:

1. Kawamorita N, Yoshikawa S, Pradeep T, Chancellor MB, Yoshimura N: Antisense oligonucleotide targeting NGF attenuates bladder pain behavior in rat with experimental colitis. 43rd Annual meeting of the International Continence Society, Abstract No. 148, Barcerona, Spain, August 26-30, 2013. Neurourology and Urodynamics (2013) 32: 727-728.
2. Oguchi T, Yokoyama H, Funahashi Y, Yoshikawa S, Nishizawa O, Goins WF, Goss JR, Glorioso JC, Chancellor M, Tyagi P, Yoshimura N: Inhibition of bladder hypersensitivity by interleukin 4 (IL-4) gene therapy using herpes simplex virus (HSV) vectors in rats with cyclophosphamide induced cystitis. 43rd Annual meeting of the International Continence Society, Abstract No. 150, Barcerona, Spain, August 26-30, 2013. Neurourology and Urodynamics (2013) 32: 729-730.

V. Conclusions

In the first year of the project, we successfully accomplished the goals outlined in the proposal and listed in the statement of work (year 1). Based on the results obtained in the first funding period, we have evaluated optimized, begun production, and established SOPs and controls for LP-11. We also laid the groundwork for future experimental testing in diseased animal models at the University of Pittsburgh. In the second year of the project, we will continue to develop methods to progress a full cGMP product and conduct animal studies according to our SOW (year 2).

Down-Regulation of Nerve Growth Factor Expression in the Bladder by Antisense Oligonucleotides as New Treatment for Overactive Bladder

Mahendra Kashyap,* Naoki Kawamorita,* Vikas Tyagi, Yoshio Sugino, Michael Chancellor,† Naoki Yoshimura and Pradeep Tyagi‡,§

From the Departments of Urology, University of Pittsburgh, Pittsburgh, Pennsylvania, and William Beaumont Oakland University School of Medicine (VT, MC), Royal Oak, Michigan

Purpose: Nerve growth factor over expression in the bladder has a role in overactive bladder symptoms via the mediation of functional changes in bladder afferent pathways. We studied whether blocking nerve growth factor over expression in bladder urothelium by a sequence specific gene silencing mechanism would suppress bladder overactivity and chemokine expression induced by acetic acid.

Materials and Methods: Female Sprague-Dawley® rats anesthetized with isoflurane were instilled with 0.5 ml saline, scrambled or TYE™ 563 labeled antisense oligonucleotide targeting nerve growth factor (12 μM) alone or complexed with cationic liposomes for 30 minutes. The efficacy of nerve growth factor antisense treatments for acetic acid induced bladder overactivity was assessed by cystometry. Bladder nerve growth factor expression levels and cellular distribution were quantified by immunofluorescence staining and enzyme-linked immunosorbent assay. Effects on bladder chemokine expression were measured by Luminex® xMAP® analysis.

Results: Liposomes were needed for bladder uptake of oligonucleotide, as seen by the absence of bright red TYE 563 fluorescence in rats instilled with oligonucleotide alone. At 24 hours after liposome-oligonucleotide treatment baseline bladder activity during saline infusion was indistinct in the sham and antisense treated groups with a mean ± SEM intercontraction interval of 348 ± 55 and 390 ± 120 seconds, respectively. Acetic acid induced bladder overactivity was shown by a decrease in the intercontraction interval to a mean of 33.2% ± 4.0% of baseline in sham treated rats. However, the reduction was blunted to a mean of 75.8% ± 3.4% of baseline in rats treated with liposomal antisense oligonucleotide ($p < 0.05$). Acetic acid induced increased nerve growth factor in the urothelium of sham treated rats, which was decreased by antisense treatment, as shown by enzyme-linked immunosorbent assay and reduced nerve growth factor immunoreactivity in the urothelium. Increased nerve growth factor in bladder tissue was associated with sICAM-1, sE-selectin, CXCL-10 and 1, leptin, MCP-1 and vascular endothelial growth factor over expression, which was significantly decreased by nerve growth factor antisense treatment ($p < 0.01$).

Conclusions: Acetic acid induced bladder overactivity is associated with nerve growth factor over expression in the urothelium and with chemokine up-regulation. Treatment with liposomal antisense suppresses bladder overactivity, and nerve growth factor and chemokine expression. Local suppression of nerve growth factor in the bladder could be an attractive approach for overactive bladder. It would avoid the systemic side effects that may be associated with nonspecific blockade of nerve growth factor expression.

Key Words: urinary bladder, overactive; nerve growth factor; urothelium; chemokines; liposomes

Abbreviations and Acronyms

AA = acetic acid
BO = bladder overactivity
CMG = cystometrogram
ICI = intercontraction interval
MCP-1 = monocyte chemoattractant protein-1
NGF = nerve growth factor
OAB = overactive bladder
OND = oligonucleotide
PBS = phosphate buffered saline
sE-selectin = soluble endothelial adhesion molecule
sICAM-1 = soluble intracellular adhesion molecule
VEGF = vascular endothelial growth factor

Accepted for publication February 20, 2013.

Study received animal care and use committee approval.

Supported by National Institutes of Health DK057267 and DK088836, and Department of Defense W81XWH-11-1-0763 and W81XWH-12-1-0565.

* Equal study contribution.

† Financial interest and/or other relationship with Allergan, Astellas, Covic, Lipella, Medtronic and Pfizer.

‡ Correspondence: Department of Urology, University of Pittsburgh, Pittsburgh, Pennsylvania (telephone: 412-692-4119; FAX: 412-692-4380; e-mail: tyagip@upmc.edu).

§ Financial interest and/or other relationship with Astellas.

0022-5347/13/1902-0757/0
THE JOURNAL OF UROLOGY®

http://dx.doi.org/10.1016/j.juro.2013.02.090
Vol. 190, 757-764, August 2013
Printed in U.S.A.

PREVIOUS studies by various groups demonstrated that NGF over expression in the bladder and bladder afferent pathways is involved in the emergence of hyperexcitability in bladder C-fiber sensory pathways.[1,2] C-fiber hyperexcitability is considered responsible for symptoms common to OAB.[3] Intrathecal application of NGF antibodies decreased NGF levels in bladder afferent pathways and normalized bladder/urethral function in spinal cord injured rats.[4]

Recently, exogenous over expression of NGF in the urothelium was shown to cause micturition dysfunction and pelvic hypersensitivity in a transgenic mouse model.[2,5] The major role of NGF in lower urinary tract symptoms encouraged the systemic administration of monoclonal human NGF antibodies in patients with mixed results.[6] In patients generalized blockade of NGF at sites other than the bladder was associated with the incidence of paresthesia, hypoesthesia and arthralgia.

Therefore, to decrease the intrinsic toxicity of systemic blockade of NGF, we developed what is to our knowledge a novel intravesical therapy for OAB by targeting intracellular synthesis of NGF in the urothelium. We measured the functional efficacy of liposomes complexed with antisense OND targeting NGF, including the effect on the NGF signaling pathway.[7–10]

METHODS

Reagents

The 18mer phosphorothioated antisense OND was custom made. It had a 5′ tag of TYE 563 (bright red fluorescent dye that is a direct substitute for Cy3™) with the sequence 5′GCCCGAGACGCCTCCCGA3′. A similar length scrambled sequence 5′ACGACCTCGCGACCGGCC3′ was designed using GenScript (https://www.genscript.com/ssl-bin/app/scramble). Cationic liposomes composed of DOTAP were made by the thin film hydration method, in which lipid film was hydrated with nuclease-free water with a final lipid concentration of 7 mM. Lyophilized OND was dispersed in nuclease-free water at a concentration of 12 μM. It was then complexed with liposomes by incubating the 2 entities together at room temperature for 30 minutes. The molar ratio of OND to lipid in the liposomal complex was 1:10.

Animals

The study was performed in 37 female adult Sprague-Dawley rats weighing 225 to 250 gm divided into 6 study groups of 5 to 8 each. We used 2 group for bladder uptake and the remaining 4 for CMG, which also provided tissue for subsequent immunohistochemistry, NGF and chemokine analysis.

Studies

Bladder uptake. Rats were anesthetized with 2% isoflurane. The bladder was catheterized by a 24 gauge angiocatheter (BD™) and washed with saline to instill 0.5 ml fluorescent TYE conjugated OND in saline (6 rats) or complexed with liposomes for 30 minutes (6 rats). A purse-string suture was placed around the urethra to occlude for 30 minutes, which was later withdrawn to restart voiding by awake rats in metabolic cages at the end of instillation. Rats were sacrificed 8 and 24 hours (3 per time point per group) after instillation to harvest bladder tissue for cryosectioning into 8 μm cryosections.

Efficacy. Rats were instilled with 12 μM NGF antisense (6) or scrambled OND complexed with liposome (5), or with saline as sham treatment (8) after the described bladder uptake studies. CMG was done 24 hours later in the 4 groups, including a control group of 6 rats without prior instillation, under anesthesia using urethane (1.0 gm/kg subcutaneously). A polyethylene-50 catheter was connected by a 3-way stopcock to a pressure transducer and to a syringe pump. The catheter was inserted in the bladder through the dome to record intravesical pressure and infuse solutions into the bladder. Intravesical pres-

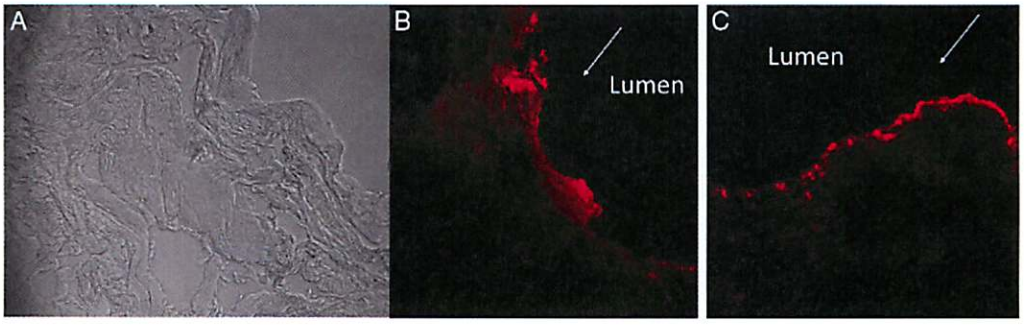


Figure 1. Confocal images show harvested rat bladders instilled with antisense OND with 5′ tag of TYE 563 without liposome (*A*), and with OND complexed with liposomes at 8 (*B*) and at 24 (*C*) hours. Bright red fluorescence demonstrates successful uptake and retention in target cells of OND delivered by liposomes (*B* and *C*). Fluorescence was more homogenous in urothelium at 24 hours vs discrete localization to lumen surface at 8 hours. Arrow indicates lumen side. Bright field image is also shown since there was no fluorescent signal for image to be taken for group instilled with antisense OND with 5′ tag of TYE 563 without liposome (*A*). Absence of red fluorescence reveals liposome need for successful bladder uptake of OND. Reduced from ×40.

sure was recorded with Chart™ data acquisition software at a sampling rate of 400 Hz on a computer system equipped with an analog-to-digital converter. Body temperature was maintained in the physiological range with a heat lamp.

Control CMG was performed by filling the bladder with saline at 0.04 ml per minute to elicit repetitive voiding for more than 1 hour. Subsequently, bladder irritation was induced by 0.25% AA infusion for more than 3 hours to induce BO, which is considered similar to the phenotype of OAB observed clinically. The ICI of reflex bladder contractions during saline and AA infusion was determined as the time between 2 continuing contraction cycles. We compared the average of at least 3 ICIs measured for more than 30 minutes during saline infusion and 60 minutes after AA infusion, respectively.

Immunohistochemistry

At the end of CMG, a portion of the bladders (5 preparations) from each group were cryopreserved. Cryosections (8 μm) were washed in PBS and preincubated with PBS containing 20% normal serum (Jackson ImmunoResearch, West Grove, Pennsylvania) and 0.2% Triton™ X-100 for 2 hours at room temperature. Primary polyclonal rabbit H-20 antibody (1:50) (Santa Cruz Biotechnology, Santa Cruz, California) for NGF was applied in PBS containing 5% normal serum and 0.2% Triton X-100 for 16 to 18 hours at 4C. Sections were washed 4 times in PBS containing 0.1% bovine serum albumin and 0.1% Triton X-100 for 5 minutes each at room temperature. They were then incubated for 2 hours at room temperature with secondary donkey anti-rabbit Alexa Fluor® 488 antibody (1:200). Washing was performed 3 times at room temperature in PBS. Sections were mounted with aqueous mounting medium.

Harvested Bladder NGF

At the end of CMG, mucosa containing the urothelium was surgically separated from the detrusor in a portion of rat bladder from each group (5 preparations), as previously described.[11] Tissues were homogenized using the RIPA Lysis Buffer System (Santa Cruz Biotechnology) to isolate protein and measure NGF using antigen capture enzyme-linked immunosorbent assay with the Emax® ImmunoAssay System according to manufacturer instructions, as previously described.[12] Tissue NGF values are expressed as pg/mg protein. Total RNA was isolated from whole bladders in control and antisense treated rats. It was later transcribed into cDNA to measure NGF mRNA levels by quantitative polymerase chain reaction, as previously described.[13]

Effect on Downstream Effectors of NGF Signaling Pathway

Tissue lysates prepared from whole bladder (5 preparations) were analyzed for the chemokines sICAM-1,[14] sE-selectin, MCP-1,[9] VEGF,[8] leptin,[7] and CXCL-1[9] and 10 using the Luminex xMAP kit, as previously described.[9]

Statistical Analysis

Results are shown as the mean ± SEM. Statistical significance between the mean values of different groups was analyzed using 1-way ANOVA, followed by the Tukey post test. The correlation of NGF and chemokine expression was assessed by the Pearson r and Spearman r_s correlation tests. In all statistical tests the minimum criterion chosen to discard the null hypothesis was set at $p < 0.05$.

RESULTS

Bladder Uptake

Rat bladders harvested after OND instillation with or without liposomes were cryosectioned for viewing under a LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany). The fluorescent signal was used as a measure of bladder uptake of OND after instillation. The need for liposomes in successful OND delivery was demonstrated by the lack of bright red fluorescence from the TYE 563 tag of OND in the absence of liposomes (fig. 1, *A*). Figure 1,

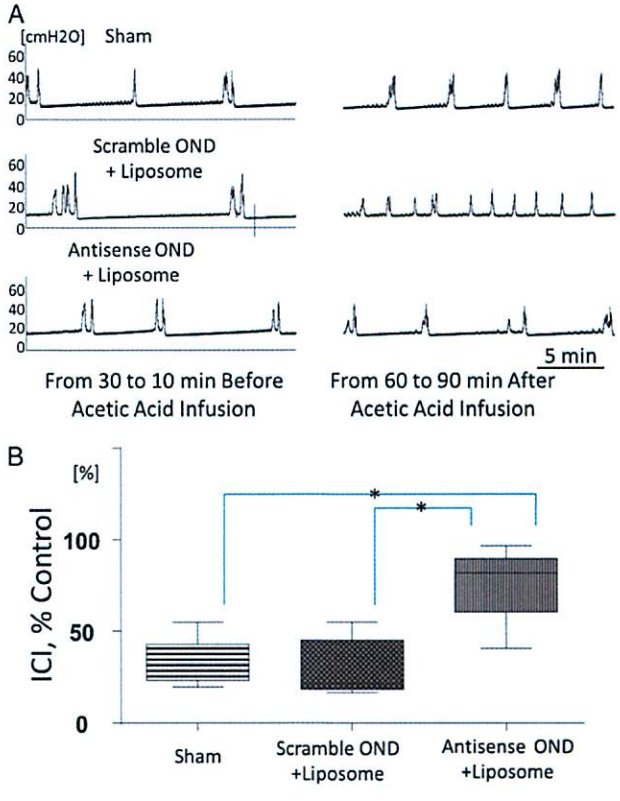


Figure 2. CMG analysis of bladder overactivity in treated groups induced by intravesical application of 0.25% AA. Representative CMG was performed 24 hours after instillation of saline in sham treated group (*Sham*), liposomal complex of scrambled OND or antisense OND (*A*). Note CMG traces 30 to 10 minutes before and 60 to 90 minutes after AA application. AA-induced reduction in ICI was seen in sham and scrambled OND treated rats but not in group treated with liposomal NGF antisense complex which demonstrates antisense OND protective effect. CMG parameters in absence of AA did not significantly differ in 3 groups. Changes in ICI after intravesical AA application expressed as percent of control ICI before AA (*B*). AA induced ICI decrease was significantly smaller in liposome-NGF antisense treated group vs sham and liposome-scrambled OND treated groups. Asterisk indicates $p < 0.05$.

B and *C* show the bright field image since there was no fluorescent signal in the images from that group. Liposomes mediated bladder uptake of OND was evident due to intense fluorescence at 8 hours (fig. 1, *B*). Penetration depth was restricted to the urothelium but it increased from 8 to 24 hours (fig. 1, *C*). Fluorescence localization in the urothelium revealed successful bladder uptake and retention of OND in target cells.

Cystometry

Baseline CMG under saline infusion was indistinct between the groups, as evident by the mean ICI of 348 ± 55 and 390 ± 120 seconds in the sham and liposomal antisense treated groups, respectively. AA induced BO was evident in the sham treated group due to the mean percent reduction to 33.2% ± 4.0% of baseline ICI (fig. 2, *A*). Pretreatment with antisense OND complexed with liposomes blocked AA induced BO with the mean percent reduction restored to 75.8% ± 3.4% of baseline (6 preparations) (fig. 2, *A*). Sequence specificity of NGF antisense was shown by the lack of effect in the group instilled with scrambled OND sequence complexed with liposomes (fig. 2, *A*). ICI was longer in the liposomal antisense treated group than in the sham treated group. Differences were statistically significant (1-way ANOVA followed by the Tukey post test $p < 0.05$, fig. 2, *B*).

Bladder NGF

AA exposure increased NGF production in the sham treated group relative to controls. Pretreatment with NGF antisense OND significantly blocked AA induced NGF over expression in the urothelium as well as in detrusor lysates (fig. 3, *A* and *B*). Changes in NGF protein expression varied in scrambled OND treated rats, resulting in insignificant changes in NGF protein expression. However, it tended to decrease, as shown by the plot of individual values

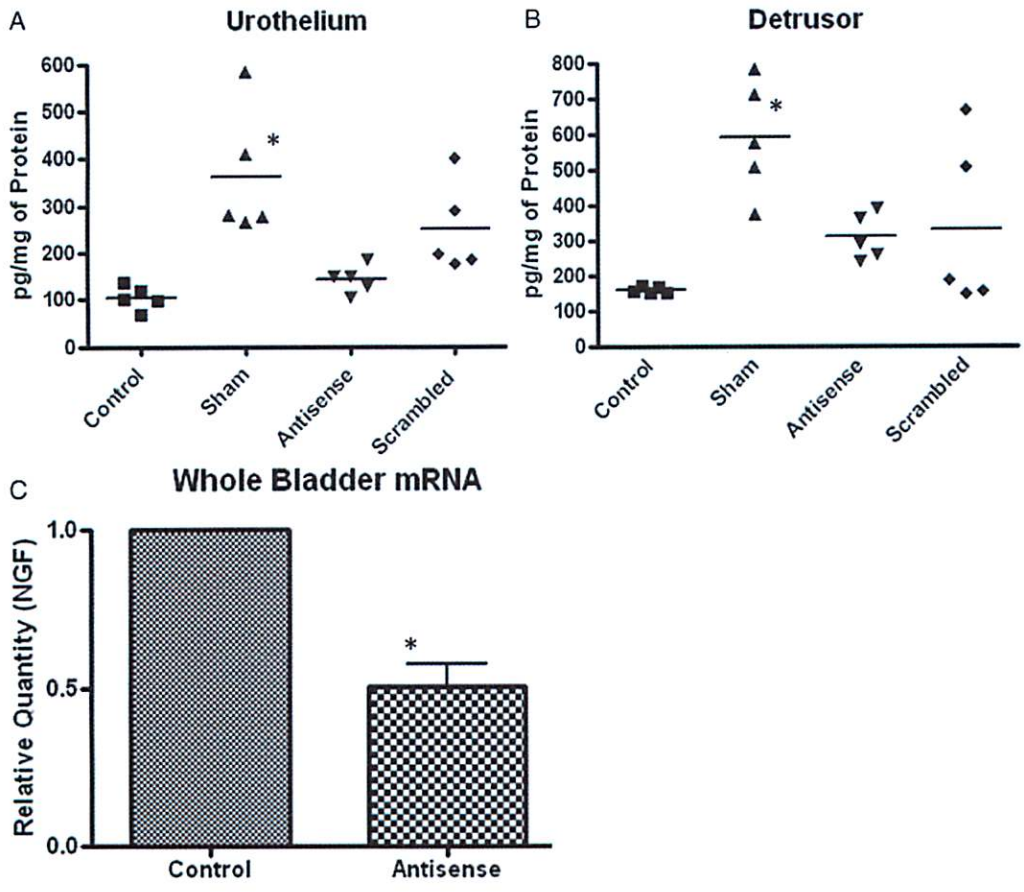


Figure 3. Antisense OND mediated suppression of AA induced NGF protein expression in urothelium (*A*) and detrusor (*B*), and NGF mRNA expression in whole bladder (*C*). AA exposure caused significant increase in NGF in sham treated group (*Sham*) vs untreated controls. NGF antisense OND significantly blunted NGF increase vs sham treated group. Data points represent NGF values of individual rats around mean (horizontal line) of each group. Significant decrease was noted in whole bladder mRNA levels of rats treated with NGF antisense OND complexed with liposomes vs levels in controls (*C*). Normalized NGF levels were compared by ANOVA followed by Tukey test. Asterisk indicates $p < 0.05$.

for each group (fig. 3, *A* and *B*). Results could be directly inferred from the urothelial uptake of OND facilitated by liposomes (fig. 3, *A*), while the effect on detrusor NGF levels may be explained by the downstream effect of NGF expressed in the urothelium.[2]

NGF mRNA levels were measured in controls. NGF transcript levels in the urothelium were around 25% higher than in the detrusor (data not shown). Tissue damage from 3-hour exposure to AA during CMG hindered successful isolation of mRNA from the bladder tissue of treated groups, except for the control and antisense OND treated groups. A significant reduction in NGF transcript levels was observed in rats treated with NGF antisense OND complexed with liposomes compared to controls ($p < 0.05$, fig. 3, *C*).

Effect on Downstream Effectors of NGF Signaling Pathway

Measurement of tissue lysates from different groups revealed that AA exposure increased the expression of chemokines activated by NGF, including sICAM-1, sE-selectin, CXCL-1 and 10, leptin, MCP-1 and VEGF (figs. 4 and 5). sE-selectin and sICAM-1 are adhesion molecules expressed by chemokine activated endothelial cells to mediate leukocyte and lymphocyte adhesion. The involvement of NGF in chemokine expression was shown by an increase in chemokines with scrambled OND and a significant reduction with liposomal NGF antisense (ANOVA followed by the Tukey test $p < 0.05$, $p < 0.01$ or $p < 0.001$). Except for the high leptin levels in sham treated rats, the levels of other chemokines were highest for scrambled OND. AA induced over expression of other chemokines, including sICAM-1, sE-selectin, CXCL-1 and 10, VEGF and MCP-1, was further increased in the scrambled OND treated group vs the sham treated group. This did not rule out any nonspecific induction of chemokine expression by scrambled OND.

When NGF levels were plotted against chemokine levels regardless of treatment group, we noted a positive association with VEGF (Pearson $r = 0.75$, $p < 0.001$) and a negative association with sICAM-1 (Spearman $r_s = -0.5$, $p < 0.01$, fig. 3, *A* and *B*). Separate analysis of higher chemokine levels in the scrambled OND group revealed that, apart from VEGF, MCP-1 also positively correlated with NGF ($r = 0.88$, $p < 0.01$).

NGF Immunostaining

Immunoreactivity for NGF (green staining) was noted in the detrusor region in all groups and it was absent in the apical cells of bladder mucosa containing urothelium untreated with AA (fig. 6, *A*). NGF immunoreactivity appeared to increase several fold in the detrusor. Its presence was distinctly identified in mucosa containing urothelium after AA infusion in the sham treated group (fig. 6, *C*). AA induced NGF immunoreactivity in the urothelium was also noted in rats instilled with scrambled OND (fig. 6, *B*). In contrast, NGF immunoreactivity in the urothelium, including a subpopulation of suburothelial cells, was decreased to levels in controls by pretreatment with liposomal antisense OND (fig. 6, *A* and *D*). Control sections incubated in the absence of primary or secondary antibody were evaluated for specificity or background staining. In the absence of primary antibody, no positive immunoreactivity was observed.

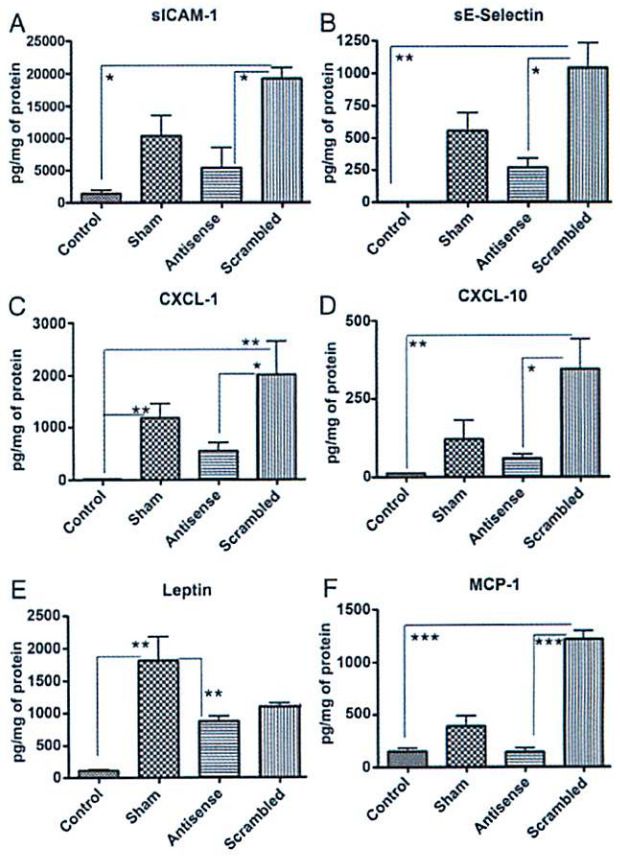


Figure 4. Effect of antisense OND on chemokines activated by NGF signaling pathway. NGF antisense OND significantly reduced chemokine over expression (*A* to *F*). Except for leptin, which was highest in sham treated rats (*Sham*), chemokine levels were highest in rat bladders instilled with scrambled sequence OND complexed with liposomes. Bars represent mean ± SEM. Significance of differences was determined vs sham or scramble treated rats by ANOVA followed by Tukey test. Single asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$.

DISCUSSION

The bladder is presumed to be the tissue source responsible for increased NGF in the urine of pa-

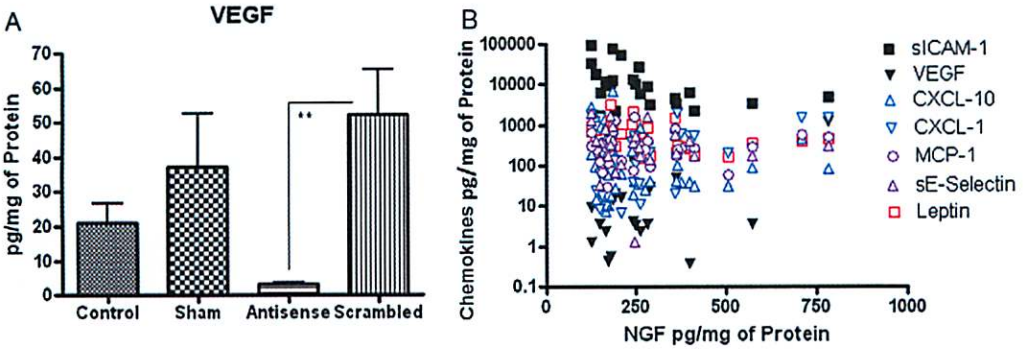


Figure 5. Antisense OND effect on VEGF (*A*) and NGF plotted with chemokines regardless of treatment group (*B*). VEGF downstream expression was significantly reduced by antisense OND (*A*). Decreased VEGF was positively associated with NGF across all treatment groups (Pearson r = 0.75, p <0.001) (*B*). sICAM-1 was negatively associated with NGF levels regardless of treatment group (Spearman $r_s = -0.5$, p <0.01). When only NGF in scrambled group was plotted against respective chemokines, MCP-1 together with VEGF positively correlated with NGF. Asterisks indicate Pearson r = 0.88, p <0.01 (*A*).

tients with OAB or interstitial cystitis/painful bladder syndrome compared to controls.[15] Since NGF over expression in the bladder is implicated as the mediator of symptoms associated with OAB, NGF can be blocked directly by antibodies[6] or indirectly by halting the translation of NGF mRNA with sequence specific gene silencing (antisense).

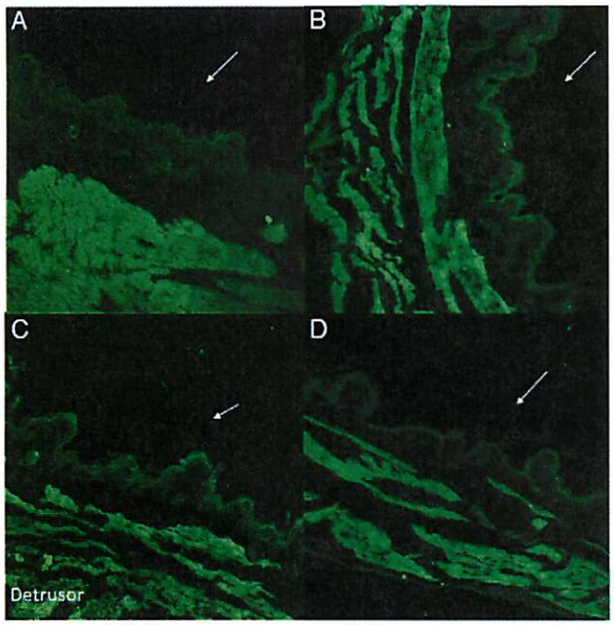


Figure 6. Representative immunofluorescence labeling in rat bladder cross sections reveals that density of NGF immunoreactivity (green areas) was highest in detrusor in all groups. It increased after AA treatment in accordance with NGF expression in muscle. NGF immunoreactivity in bladder mucosa containing urothelium cell layer was absent in rats untreated with AA (*A*). It was noted in sham (*C*) and scrambled OND (*B*) treated rats. NGF immunoreactivity in urothelial cell layer was reduced in rats treated with antisense OND complexed with liposomes (*D*) to levels comparable to controls (*A*). Reduced from ×20.

The primary impediment to developing intravesical antisense therapy is inefficient bladder uptake of OND across the urothelium. Bladder uptake of fluorescent OND without liposomes is deficient due to poor intracellular passage of OND across the urothelium (fig. 1, *A*). Uptake of anionic OND is probably limited by the size of the OND and the charge interaction with the urothelial anionic glycosaminoglycan layer. Our prior studies of liposome interaction with urothelial cells showed liposome adsorption and endocytosis.[16] Cationic liposomes were successfully used to deliver siRNA after intravesical administration in the murine bladder.[17] The current study supports the use of cationic liposomes as an OND carrier.

The rapid increase in NGF protein levels noted in sham treated rats after AA exposure could be blunted by pretreatment with NGF antisense OND (figs. 3 and 6, *C*). Down-regulation of NGF mRNA expression is in agreement with decreased protein levels and suppressed NGF-like immunoreactivity in the urothelium (figs. 3, *C* and 6, *D*). CMG data, NGF levels and chemokine suppression together support our hypothesis that NGF released from the urothelium is an important chemical mediator responsible for changes in bladder function (figs. 2 to 6).[18]

Rats pretreated with scrambled OND were devoid of any functional treatment response on CMG or any chemokine suppression (figs. 2, 4 and 5). However, scrambled OND was associated with variable but insignificantly reduced NGF protein compared to sham treated levels (fig. 3, *A* and *B*). The disparity in chemokine and NGF levels in the treated groups indicates interaction between chemokine and NGF

expression in the bladder after intravesical AA infusion. Increased chemokine expression with scrambled OND indicates aggravated tissue inflammation, which may cause mRNA and protein decay. mRNA degradation may partially explain the inability to perform mRNA analysis in some groups, although it is unclear whether protein degradation had a role in the reduced NGF protein levels in different groups (fig. 3). Future studies in animals that over express the NGF gene may clarify the disparity in NGF and chemokine levels caused by antisense OND.

Increased NGF expression in the urothelium and detrusor combined with dense NGF immunoreactivity in the detrusor confirmed them as 2 major sources of bladder NGF (figs. 3, *A* and *B*, and 6).[19] Considering our bladder uptake data (fig. 1), we cannot rule out the modulation of detrusor NGF levels by freshly produced NGF in the urothelium.[10] NGF immunoreactivity in the absence of AA exposure may be due to receptor bound NGF that is not available to act on the detrusor. The decrease in NGF protein levels and immunoreactivity after antisense treatment is similar to the decreased urinary excretion of NGF protein in patients with OAB after antimuscarinic or Botox® therapy.[11,20]

It was previously reported that BO caused by exposure to irritants (turpentine), akin to the AA induced BO that we studied, involves a rapid increase in the bladder content of NGF, which occurs within 2 hours of irritant exposure.[21] Later studies showed that within 30 minutes of exposure to insults such as lipopolysaccharide, the bladder responds by up-regulating the genes of NGF and sE-selectin, and the receptor of MCP-1.[10] These earlier results agree with our findings of the reported increase in the protein levels of NGF, MCP-1, sE-selectin, sICAM-1, leptin, CXCL-1 and 10, and VEGF in bladder tissue after 3-hour AA exposure (figs. 3 to 5).

NGF is a paracrine messenger involved in physiological and pathological signaling[2] that activates several downstream effectors to manifest signaling changes.[7,8,10,14] Chemokines are one of several downstream effectors activated by NGF.[7,8,10,14] Interestingly, chemokine receptors are widely expressed in neural and nonneural elements of the nociceptive pathways responsible for visceral and somatic pain sensation.[22] MCP-1[23] and CXCL-10[24] are constitutively expressed in neurons (fig. 4, *D* and *F*), where they participate in the excitability of primary afferent neurons via transactivation of transient receptor channels and nociceptor sensitization.[24] Chemokine localization in neuron synaptic vesicles is consistent with their ability to act as excitatory neurotransmitters after AA exposure.[25]

It was recently reported that NGF binding to its high affinity TrkA receptor controls sICAM-1 expression on target cells[14] and inhibition of NGF expression significantly down-regulates ICAM-1 expression.[14] The negative association of NGF with sICAM-1 in all treatment groups corroborates the reported regulating effect of NGF on sICAM-1 expression (figs. 4, *A* and 5, *B*).[14] The reported activation of VEGF expression by NGF[8] is consistent with the positive association of VEGF and NGF levels in all treatment groups (fig. 5, *B*).

AA exposure is not known to selectively induce NGF expression and the involvement of other mediators, such as prostaglandins,[26] in the BO model tested cannot be ruled out. The role of prostaglandins in AA induced BO may explain the variable expression of chemokines in the different groups. Prostaglandins induce mRNA coding for CXCL-1[27] and MCP-1,[28] while at the same time decreasing leptin mRNA levels (fig. 4, *C*, *E* and *F*).[28] NGF dependent leptin expression[7] is presumed to emerge from bladder adipocytes associated with afferent neurons.[29] Compared to the short biological half-life of prostaglandins, chemokines are long acting downstream effectors and may be better suited to track treatment response in tissue or urine.[9,30]

Taken together, our data support the hypothesis that increased bladder NGF content after AA irritation can be blocked by local instillation of antisense OND complexed with liposomes. These observations are consistent with the presumed role of NGF in OAB symptoms.[20] However, to our knowledge it remains to be determined how NGF expression blockade in the urothelium affects the excitability of bladder afferents leading to BO. Future steps in the drug development of this strategy will be the duration of effect and the effect in other models of bladder irritation and overactivity.

CONCLUSIONS

NGF down-regulation as novel treatment for BO also suppresses the downstream signaling cascades activated by NGF leading to reduced chemokine expression. The intravesical route is the most appropriate choice for anti-NGF therapy since the bladder is the putative source of NGF responsible for increased C-fiber afferent nerve excitability and BO. Liposomes represent a delivery platform for the local delivery of antisense based therapy that may avoid systemic toxicity.

ACKNOWLEDGMENTS

OND was made at Integrated DNA Technologies, San Diego, California.

REFERENCES

1. Yoshimura N and de Groat WC: Increased excitability of afferent neurons innervating rat urinary bladder after chronic bladder inflammation. J Neurosci 1999; **19:** 4644.
2. Schnegelsberg B, Sun TT, Cain G et al: over expression of NGF in mouse urothelium leads to neuronal hyperinnervation, pelvic sensitivity, and changes in urinary bladder function. Am J Physiol Regul Integr Comp Physiol 2010; **298:** R534.
3. Ochodnicky P, Cruz CD, Yoshimura N et al: Neurotrophins as regulators of urinary bladder function. Nat Rev Urol 2012; **9:** 628.
4. Seki S, Sasaki K, Fraser MO et al: Immunoneutralization of nerve growth factor in lumbosacral spinal cord reduces bladder hyperreflexia in spinal cord injured rats. J Urol 2002; **168:** 2269.
5. Zhang YH, Vasko MR and Nicol GD: Ceramide, a putative second messenger for nerve growth factor, modulates the TTX-resistant Na(+) current and delayed rectifier K(+) current in rat sensory neurons. J Physiol 2002; **544:** 385.
6. Evans RJ, Moldwin RM, Cossons N et al: Proof of concept trial of tanezumab for the treatment of symptoms associated with interstitial cystitis. J Urol 2011; **185:** 1716.
7. Cardenas S, Scuri M, Samsell L et al: Neurotrophic and neuroimmune responses to early-life Pseudomonas aeruginosa infection in rat lungs. Am J Physiol Lung Cell Mol Physiol 2010; **299:** L334.
8. Nakamura K, Tan F, Li Z et al: NGF activation of TrkA induces vascular endothelial growth factor expression via induction of hypoxia-inducible factor-1alpha. Mol Cell Neurosci 2011; **46:** 498.
9. Tyagi P, Barclay D, Zamora R et al: Urine cytokines suggest an inflammatory response in the overactive bladder: a pilot study. Int Urol Nephrol 2010; **42:** 629.
10. Saban MR, Hellmich H, Nguyen NB et al: Time course of LPS-induced gene expression in a mouse model of genitourinary inflammation. Physiol Genomics 2001; **5:** 147.
11. Ha US, Park EY and Kim JC: Effect of botulinum toxin on expression of nerve growth factor and transient receptor potential vanilloid 1 in urothelium and detrusor muscle of rats with bladder outlet obstruction-induced detrusor overactivity. Urology 2011; **78:** 721 e1.
12. Jacobs BL, Smaldone MC, Tyagi V et al: Increased nerve growth factor in neurogenic overactive bladder and interstitial cystitis patients. Can J Urol 2010; **17:** 4989.
13. Tyagi S, Tyagi P, Van-le S et al: Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor. J Urol 2006; **176:** 1673.
14. Othumpangat S, Regier M and Piedimonte G: Nerve growth factor modulates human rhinovirus infection in airway epithelial cells by controlling ICAM-1 expression. Am J Physiol Lung Cell Mol Physiol 2012; **302:** L1057.
15. Liu HT, Tyagi P, Chancellor MB et al: Urinary nerve growth factor but not prostaglandin E2 increases in patients with interstitial cystitis/bladder pain syndrome and detrusor overactivity. BJU Int 2010; **106:** 1681.
16. Nirmal J, Tyagi P, Dang L et al: Endocytosis uptake of liposomes in urothelium cells detected by transmission electron microscopy. J Urol 2012; **187:** e15.
17. Nogawa M, Yuasa T, Kimura S et al: Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer. J Clin Invest 2005; **115:** 978.
18. Dupont MC, Spitsbergen JM, Kim KB et al: Histological and neurotrophic changes triggered by varying models of bladder inflammation. J Urol 2001; **166:** 1111.
19. Tanner R, Chambers P, Khadra MH et al: The production of nerve growth factor by human bladder smooth muscle cells in vivo and in vitro. BJU Int 2000; **85:** 1115.
20. Liu HT, Chancellor MB and Kuo HC: Decrease of urinary nerve growth factor levels after antimuscarinic therapy in patients with overactive bladder. BJU Int 2009; **103:** 1668.
21. Oddiah D, Anand P, McMahon SB et al: Rapid increase of NGF, BDNF and NT-3 mRNAs in inflamed bladder. Neuroreport 1998; **9:** 1455.
22. White FA, Jung H and Miller RJ: Chemokines and the pathophysiology of neuropathic pain. Proc Natl Acad Sci U S A 2007; **104:** 20151.
23. Banisadr G, Gosselin RD, Mechighel P et al: Constitutive neuronal expression of CCR2 chemokine receptor and its colocalization with neurotransmitters in normal rat brain: functional effect of MCP-1/CCL2 on calcium mobilization in primary cultured neurons. J Comp Neurol 2005; **492:** 178.
24. Bhangoo S, Ren D, Miller RJ et al: Delayed functional expression of neuronal chemokine receptors following focal nerve demyelination in the rat: a mechanism for the development of chronic sensitization of peripheral nociceptors. Mol Pain 2007; **3:** 38.
25. Jung H, Toth PT, White FA et al: Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons. J Neurochem 2008; **104:** 254.
26. Chidiac JJ, Al-Asmar B, Rifai K et al: Inflammatory mediators released following application of irritants on the rat injured incisors. The effect of treatment with anti-inflammatory drugs. Cytokine 2009; **46:** 194.
27. Wang D, Wang H, Brown J et al: CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer. J Exp Med 2006; **203:** 941.
28. Peeraully MR, Sievert H, Bulló M et al: Prostaglandin D2 and J2-series (PGJ2, Delta12-PGJ2) prostaglandins stimulate IL-6 and MCP-1, but inhibit leptin, expression and secretion by 3T3-L1 adipocytes. Pflugers Arch 2006; **453:** 177.
29. Maeda T, Kiguchi N, Kobayashi Y et al: Leptin derived from adipocytes in injured peripheral nerves facilitates development of neuropathic pain via macrophage stimulation. Proc Natl Acad Sci U S A 2009; **106:** 13076.
30. Tyagi P, Killinger K, Tyagi V et al: Urinary chemokines as noninvasive predictors of ulcerative interstitial cystitis. J Urol 2012; **187:** 2243.

148

Kawamorita N[1], Yoshikawa S[1], Pradeep T[1], Mb C[2], Yoshimura N[1]
***1.** University of Pittsburgh, **2.** Oakland University*

ANTISENSE OLIGONUCLEOTIDE TARGETING NGF ATTENUATES BLADDER PAIN BEHAVIOR IN RAT WITH EXPERIMENTAL COLITIS

Hypothesis / aims of study

Pelvic organ "cross sensitization" is suspected to contribute to clinically overlapping symptoms in patients with chronic pelvic pain syndrome (CPPS) such as irritable bowel syndrome (IBS) and bladder pain syndrome/interstitial cystitis (BPS/IC). Previous animal studies demonstrated that experimental colitis evoked bladder overactivity associated with hyperexcitability of afferent neurons innervating the bladder [1] although it has not been investigated whether this colitis model exhibits bladder pain. On the other hand, overexpression of nerve growth factor (NGF) in the bladder has been shown to play an important role in the symptom development in BPS/IC patients. We recently reported that instillation of liposome conjugated with antisense oligonucleotide (OND) targeting NGF into the bladder suppressed bladder overactivity in a rat model of acute cystitis. Therefore, this study was performed to explore whether pain behaviour induced by bladder irritation and NGF expression in the bladder are increased after colitis and whether instillation of liposomal-ODN conjugates into the bladder can suppress pain behaviour and NGF expression in a rat model of experimental colitis.

Study design, materials and methods

Female Sprague-Dawtey rats were used and divided into five groups; (a) control group (no treatment), (b) colitis-OND group (intracolonic 2,4,6,trinitrobenzen sulfonic acid [TNBS] enema and intravesical liposomal OND were given) , (c) colitis-saline group (intracolonic TNBS and intravesical saline were given), (d) sham-ODN group (intravesical liposomal ODN was given without colitis) and (e) sham-saline group (intravesical saline was given without colitis). NGF antisense-liposome solution for intravesical application was made by dissolving 2µl of anti- NGF ODN (2mM) in 0.5ml of liposome (7mM). Under isoflurane anethesia, 0.5ml of either liposomal-ODN or saline was instilled to the bladder through an inserted urethral catheter. Twenty-four hours after instillation of liposomal-ODN or saline and fasting, colitis was induced by the enema of 30mg TNBS dissolved in 50% ethanol through a polyethylene catheter inserted 8 cm proximal to the anus in a head-down position. Ten days after liposomal-ODN or saline injection, animals were subjected to either pain behaviour testing or bladder tissue removal.

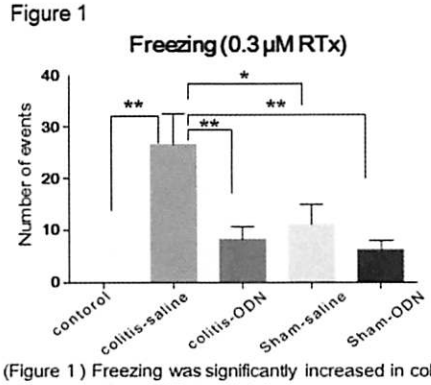
(1) Nociceptive behaviour testing: Licking and freezing behaviour in response to 1-min intravesical administration of resiniferatoxin (RTx), a TRPV1 receptor agonist was examined. After 2 hours acclimation in a metabolic cage, RTx (0.3µM, 0.3ml) was instilled through the inserted urethral catheter for 1 min and the catheter was then removed. Thereafter both licking and freezing behaviours were scored during 5-s intervals for 15 minutes in the cage (n=4-6).

(2) Quantification of messenger RNA (mRNA) and protein of NGF: The harvested bladder was microdissected to divide to mucosal and detrusor layers. Quantitative polymerase chain reaction (qPCR) and Enzyme-Linked ImmunoSorbent Assay (ELISA) were used to measure the mRNA and protein expression of NGF, respectively (n=5).

Results

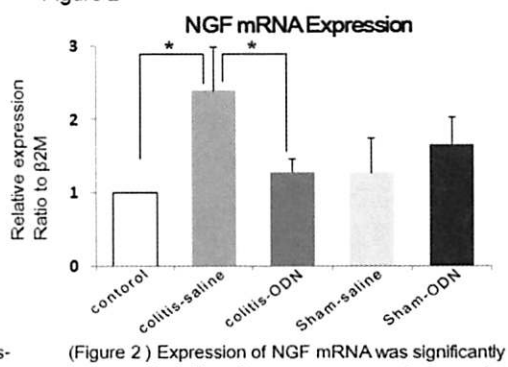
(1) In the colitis-saline group, the score of freezing behaviour was significantly higher than that of all other groups including the colitis-ODN group (Figure 1). The licking score in the colitis-saline group was significantly higher than in the control group and tended to higher compared to other 3 groups without significant differences. (2) The mRNA expression of NGF in the colitis-saline group was significantly increased in the mucosa compared to control and colitis-OND groups (Figure 2). In addition the protein level of NGF in the mucosa was also higher in the colitis-saline group compared to other groups.

Figure 1



(Figure 1) Freezing was significantly increased in colitis-saline group (*p<0.05, **p< 0.01).

Figure 2



(Figure 2) Expression of NGF mRNA was significantly increased in colitis-saline group compared to control and colitis-ODN group (*p<0.05).

Interpretation of results

Colitis evoked by TNBS enhanced the freezing behaviour, which corresponds to bladder pain [2], and increased NGF expression at both mRNA and protein levels in the bladder mucosa. These results indicate that bowel inflammation facilitates the nociceptive responses derived from the bladder in association with the increased expression of NGF in the bladder and that the intravesical instillation of NGF antisense with liposome reduces the bladder pain behaviour and the mucosal expression of NGF. Thus, it seems likely that NGF overexpression in the bladder has an important role in the colon-to-bladder cross-

sensitization to induce bladder pain after colitis and that intravesical application of liposomal ODN targeting NGF is effective to reduce NGF production in the bladder and bladder pain sensation induced by colitis.

Concluding message

This study shows that the rat model of experimental colitis is useful to study the mechanism inducing bladder pain behaviour in addition to bladder overactivity that has previously been shown [2] The liposomal antisense treatment targeting NGF in the bladder could be a new, effective modality for the treatment of bladder pain in CPPS patients including those with BPS/IC and IBS, in whom the cross-sensitization mechanism is involved in the emergence of overlapping symptoms from different pelvic organs.

References

1. Gastroenterology. 2005 Jun;128(7):1953-64
2. Neurourol Urodyn. 2010 Mar;29(3):501-5

Disclosures

Funding: Source of Funding: NIH DK088836, DOD W81XWH-11-1-0763 and W81XWH-12-1-0565 **Clinical Trial:** No **Subjects:** ANIMAL **Species:** Rat **Ethics Committee:** Institutional Animal Care and Use Committee of University of Pittsburgh

150

Oguchi T[1], Yokoyama H[2], Funahashi Y[3], Yoshikawa S[3], Nishizawa O[2], Goins W F[3], Goss J R[3], Glorioso J C[3], Chancellor M[4], Tyagi P[3], Yoshimura N[3]

1. Nagano Municipal Hospital, 2. Shinshu University, 3. University of Pittsburgh, 4. William Beaumont Hospital

INHIBITION OF BLADDER HYPERSENSITIVITY BY INTERLEUKIN 4 (IL-4) GENE THERAPY USING HERPES SIMPLEX VIRUS (HSV) VECTORS IN RATS WITH CYCLOPHOSPHAMIDE INDUCED CYSTITIS

Hypothesis / aims of study

Painful bladder syndrome/interstitial cystitis (PBS/IC) is a serious disease whose main symptoms are bladder pain and frequent urination. We first examined whether chemical cystitis induced by cyclophosphamide (CYP) enhances pain behaviour elicited by bladder irritation using resiniferatoxin (RTx). Furthermore, we investigated effects of gene therapy using replication-deficient HSV vectors expressing anti-inflammatory cytokine IL-4 (S4IL4) on pain behaviour and bladder overactivity induced by intravesical application of RTx in this cystitis rat model.

Study design, materials and methods

(1) Saline or CYP200mg/kg was injected to female SD rats intraperitoneally. Two days later, in an awake condition, 0.3uM RTx (0.3ml, 1 min) was injected to the bladder through a urethral catheter to evaluate nociceptive behaviours such as licking (lower abdominal licking) and freezing (motionless head-turning) were counted and recorded every 5 seconds for 15 minutes [1]. Urine volume and frequency were recorded simultaneously in metabolic cages (n=5 each).

(2) The replication-deficient HSV vector expressing LacZ, but not IL-4, was used as a control (SHZ). Two weeks prior to study, 20 μl of viral suspension (3.9×10^9 pfu/ml S4IL4 or 5×10^8 pfu/ml SHZ) were injected to the bladder wall. CYP200mg/kg was injected to HSV infected rats 2 days before the study. In an awake condition, 0.3uM RTx (0.3ml, 1 min) was injected to the bladder to evaluate nociceptive behaviours (licking and freezing). Urinary frequency was also compared (n=6 each).

Results

(1) Freezing behaviour, which corresponds to bladder-derived pain [1], was significantly increased in CYP cystitis rats compared to saline-treated control rats (72 ± 17 vs. 13 ± 5times, $p<0.001$) (Fig.1). Bladder volume (urine volume/ micturition) was significantly decreased in CYP cystitis rats compared to controls (0.2 ± 0.03 vs. 0.61 ± 0.08 ml, $p<0.01$) (Fig.2).

(2) Freezing behaviour induced by 0.3ul RTx was significantly decreased in S4IL4 rats by 72% compared to SHZ rats (24 ± 3 vs. 87 ± 10 times, $p<0.01$) (Fig.3). Also, bladder volume after RTx stimulation was significantly increased in S4IL4 rats compared to SHZ rats (0.55+-0.14 vs. 0.28+-0.08 ml, $p<0.05$) (Fig.4).

Figure 1

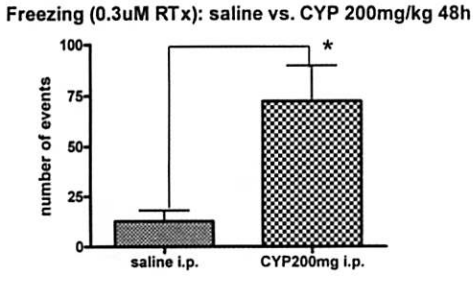


Figure 2

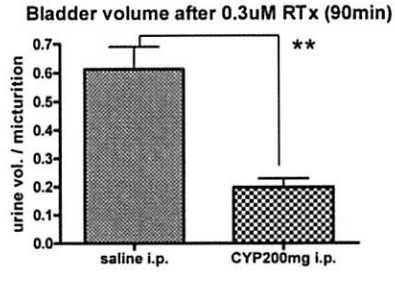


Figure 3

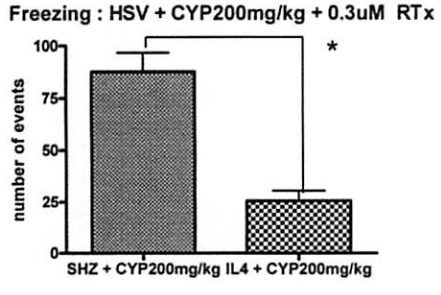
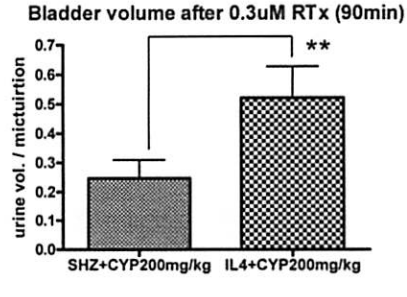


Figure 4



Interpretation of results

(1) In subacute (2 days) cystitis induced by CYP, a low concentration of RTx, which induces minimal behavioural changes in normal rats, was enough to elicit bladder pain behaviour and reduce bladder volume, indicative of bladder hypersensitivity after CYP-induced bladder inflammation.

(2) HSV vector-mediated IL-4 gene therapy suppressed bladder overactivity and enhanced pain behaviour in the subacute CYP cystitis rat model. Since we previously showed that the HSV-IL4 treatment induces IL-4 expression in the bladder and lumbosacral dorsal root ganglia (DRG) and reduces inflammatory cytokines in the bladder in an acute cystitis rat model [2], anti-inflammatory IL-4 gene therapy seems to be also effective to suppress inflammation in bladder and DRG to reduce bladder pain and overactivity in this subacute cystitis condition.

Concluding message

Bladder inflammation evokes bladder hypersensitivity and overactivity, which are suppressed by anti-inflammatory cytokine therapy mediated by replication-deficient HSV vectors. Thus, IL-4 gene therapy could be a new strategy for treating bladder pain and/or urinary frequency in patients with PBS/IC.

References

1. Saitoh C, Chancellor MB, de Groat WC et al: Effects of intravesical instillation of resiniferatoxin on bladder function and nociceptive behavior in freely moving, conscious rats. J Urol 2008; 179: 359.
2. Oguchi T, Funahashi Y, Yokoyama H et al: Effect of herpes simplex virus vector-mediated interleukin-4 gene therapy on bladder overactivity and nociception.Gene Ther. 2013 Feb;20(2):194-200.

Disclosures

Funding: NIH DK088836, P01 DK44935 and DOD W81XWH-12-1-0565 **Clinical Trial:** No **Subjects:** ANIMAL **Species:** Rat **Ethics Committee:** University of Pittsburgh Institutional Animal Care and Use Committee